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(54) Title: HYBRID PROTEIN COMPRISING T-HELPER CELL STIMULATING EPITOPES AND B-CELL EPITOPES FROM THE MAJOR OUTER MEMBRANE PROTEIN OF CHLAMYDIA TRACHOMATIS AND ITS USE AS A VACCINE

(57) Abstract

A recombinant hybrid protein is disclosed which expresses at least one chlamydial B-cell neutralizing antibody stimulating epitope and one chlamydial T-helper cell epitope, and is capable of inducing antibodies immunoreactive with *Chlamydia trachomatis* in vertebrate. Also disclosed is a nucleic acid molecule comprising at least a portion encoding the recombinant hybrid protein and an antibody immunoreactive with *C. trachomatis* producible by immunizing a host with an immunogenic component comprising the recombinant hybrid protein or the nucleic acid molecule and a carrier. The present invention relates to recombinant hybrid proteins, containing both B and T-helper cell epitopes which can be used in vaccines to provide a protective response to *C. trachomatis* serovars.

HYBRID PROTEIN COMPRISING T-HELPER CELL STIMULATING EPITOPES AND B-CELL EPITOPES FROM THE MAJOR OUTER MEMBRANE PROTEIN OF CHLAMYDIA TRACHOMATIS AND ITS USE AS A VACCINE

BACKGROUND OF THE INVENTION

(a) Field of the Invention

5   The present invention relates to a novel peptide which is of use as vaccine, to a method of preparing it and to a pharmaceutical composition containing it.   In particular, the invention relates to vaccines against *Chlamydia trachomatis*.

10   (b) Description of Prior Art

Vaccination as a deliberate attempt to protect humans against disease has a long history, although only in the 20th century has the practice flowered into routine vaccination of large populations.   During the
15   last 200 years, since the time of Edward Jenner, vaccination has controlled nine major diseases, at least in parts of the world, as follows: smallpox, diphtheria, tetanus, yellow fever, pertussis, poliomyelitis, measles, mumps, and rubella.   In the case of smallpox,
20   the dream of eradication has been fulfilled, as this disease has disappeared from the world.   Vaccinations against influenza, hepatitis B, pneumococci, and Haemophilus influenzae type b have major headway against those infections, but much remains to be done,
25   even in developed countries.

The impact of vaccination on the health of the world's peoples is hard to exaggerate.   With the exception of safe water, no other modality, not even antibiotics, has had such a major effect on mortality reduc-
30   tion and population growth.

Live vaccines have been used for some time. Protection from small pox by variolation was practiced in Europe for several hundred years prior to 1850. This practice was superseded by the use of naturally occur-
35   ring cow pox viruses discovered by Jenner. An effort to attenuate microorganisms was first made by Pasteur, who

worked with *Bacillus anthracis* and the rabies virus in the late 19th century. The BCG vaccine against tuberculosis was developed in 1921, and the yellow fever vaccine in 1937. The advent of advanced tissue culture

5   techniques in the last 40 years has promoted many of the live attenuated vaccines in current use. Most live vaccines were produced by extensive passage of virulent forms and isolating nonvirulent derivatives. Alternatively, nonvirulent forms that occur naturally are iso-

10 lated and can be used as vaccines. The Sabin virus, prepared as an attenuated vaccine, differs from the wild-type neurovirulent form of poliovirus by 57 point mutations. These mutations are distributed throughout the genome and explain the success of this vaccine.

15   It is possible to molecularly engineer microorganisms using site-directed mutagenesis or by shuffling their various regions from one serotype to another. However, there is always the difficulty of ensuring that these molecular manipulations do not interfere

20 with microorganism assembly. It is also possible to enhance attenuation by growing a microorganism in the presence of a monoclonal antibody (mAb) to a specific region.

         Live attenuated vaccines have many advantages.

25 First, they produce high levels of both humoral and cellular immunity. The problems with attenuated vaccines are the mutation and reversion to a more virulent form during the process of attenuation, which, in some cases, can be minimized by inducing several mutations

30 at distinct regions of the genome of the microorganism.

         Nevertheless, killed vaccines do have certain advantages that contrast with the theoretical shortcomings of live vaccines. By definition, killed vaccines cannot multiply or disseminate to cause the dis-

35 ease they are intended to prevent or transmit the dis-

ease to another person.   Generally, they are better
tolerated, especially for the majority of killed vac-
cines that undergo purification to remove other macro-
molecules.   Killed vaccines usually function by stimu-
5   lating humoral immune responses as well as by priming
for immunological memory.

The earliest approach to making killed vac-
cines, which is akin to the classical strategies for
making live vaccines discussed previously, relied on
10   the inactivation of whole bacteria or whole viruses
with the objective of eliciting the formation of anti-
bodies to many antigens, some of which would neutralize
the pathogen.   However, in some cases (*Chlamydia tra-
chomatis* for example) important adverse effects render
15   the use of the whole pathogen impossible and other
strategies have to be consider.

The use of protein-based or peptide-based vac-
cines represents one such strategy.

Several structures are found on the surface of
20   microorganisms; among them, some are immunogenic, that
is they are recognized by the host immune system and
may induce protective immune response.   It could be
speculated that these structures, when they are pro-
teins, can be used as a vaccine, particularly the
25   region corresponding to the immunoreactive part of the
protein.   First, the amino acid sequence of this pro-
tein must be determined by molecular biology techniques
and the antigenic determinants identified from the
amino acid sequence of a known antigen. Since some
30   antigenic sites contain charged and polar amino acids
that are readily determined by a hydrophilicity plot of
each known amino acid of the sequence, these hydrophil-
icity values can therefore be used to predict the
hydrophilicity of a particular region along the peptide
35   chain and identify the antigenic sites.   However, the

three-dimensional structure from the knowledge of the only primary sequence cannot be predicted. Predictions are complicated by the ways amino acids influence one another that are far removed from the linear sequence.

5 This can be appreciated by the techniques of Chou and Fasman, by x-ray crystallography, by nuclear magnetic resonance or by computer-aided analysis.

Another approach to define antigenic sites could be to produce a neutralizing monoclonal antibody

10 and to identify the sequential peptides bound by the antibody. It is also possible to use predictive model of the structure, for example, to define the solvent accessible areas and simply use the primary structure to predict antigenic regions.

15 While synthetic peptides constitute a very attractive approach for vaccination, due to their security and unlimited supply aspect, they are also subject to the peptide synthesis limits. In fact, it is generally admitted that the fMOC and tBOC chemistry have a

20 synthesis limit of 40 to 70 amino acid residues, depending on the instruments. It is therefore interesting to have the possibility to use a system that permit the production of longer polypeptides or proteins.

25 Molecular biology techniques offer this possibility. Moreover, a variety of hybrid constructions can also be achieved, having for result that the desired protein can be designed precisely . Using different expression systems, it is possible to chose the

30 protein form that possesses the conformation that is the most in accordance with the native protein, for example the respect of glycosylation sites and disulfide bonds.

SUMMARY OF THE INVENTION

In a first aspect the invention provides a peptide which comprises (i) a region which is able to induce the production of neutralizing antibodies to an
5 epitope of a pathogen in a host organism having an immune system which includes B-cells and T-helper cells, and (ii) a region which is able to stimulate a T-cell response to said pathogen in said organism.

Examples of suitable host organisms include
10 mammals such as humans.

Suitably region (i) comprises a B-cell epitope of said pathogen and region (ii) comprises a T-helper cell epitope of said pathogen. As used herein, the expression "B cell epitope" refers to the sequence of
15 an epitope in a pathogen which stimulates a B cell antibody production response or to a variant of this sequence which produces antibodies which cross-react with said epitope. Similarly, the expressions "T-cell epitope" or "T-helper cell epitope" refers to an epi-
20 tope in a pathogen which can stimulate a T-cell response in a host organism, or to a variant of said sequence which produces a similar response.

In a preferred embodiment, the peptide comprises two B-cell epitopes (i) and one T-helper cell
25 epitope (ii) linked in any order, preferably in a consecutive or colinear manner. The epitopes may be joined together directly or may be separated by linker sequences. The use of two B-cell epitopes will help to provide a broad protective range covering multiple
30 serovars.

In particular, the present invention provides a peptide comprising substantially only regions (i) and (ii) above i.e. peptides which predominantly comprise a region able to induce neutralizing antibodies to an
35 epitope of a pathogen and a region able to stimulate a

T-cell response to the pathogen. Thus in this particular aspect there is the drawing together of regions (i) and (ii) as set out above closely together on a relatively short peptide sequence. Of course it is impor-

5   tant that the epitopes will be correctly presented in terms of conformation for effective recognition by, and stimulation of the immune system.

Therefore the regions (i) and (ii) may be separated by linker sequences of sufficient length in order

10   to ensure optional conformation and presentation of the regions defined.

For instance, the peptide may include or consist of any of the following arrangements:

B-T-B

15

T-B-B

B-B-T

or in any combination that enhances immunogenicity, whereby "B" represents a B cell stimulating epitope and "T" represents a T helper cell stimulating epitope.

20   Identification of such regions in the case of a particular pathogen will be within the knowledge of the skilled person or may be determined by routine investigation, for example as illustrated hereinafter.

A peptide of this nature is able to induce a

25   complete and effective immune response and therefore be useful as a vaccine. The presence of B-cell epitopes induces the production of neutralizing antibodies, and T-helper cell epitopes enhance the immunogenicity of haptenic neutralizing B-cell epitopes and provide T-

30   cell memory.

The peptide as described herein may form part of a fusion protein which incorporates other peptides such as immunogenic carriers like the cholera toxin B subunit.

The peptide of the invention may be synthesized by standard solid-phase methods (fMOC of tBOC) in such a way that the final polypeptide will comprise both a neutralizable B-cell or helper T-cell target epitope.
5   Thus the invention further provides a process for preparing a peptide which comprises synthesizing an amino acid sequence which comprises an antigenic determinant or target epitope in a repetitive form.

Alternatively, it may be prepared using recombinant DNA technology as is conventional in the art.
10 For this purpose, a nucleic acid which encodes a peptide as described above is prepared using conventional techniques. This may involve a total synthesis approach or mutation by deletion of DNA extracted from
15 the relevant pathogen, or a combination of these. A particularly suitable method involves isolation of pathogenic DNA, followed by amplification of the required epitopic regions using for example the polymerase chain reaction (PCR). These regions may
20 then be ligated together using conventional techniques and incorporated into an expression vector.

Nucleic acid which encode the above-described peptide form a further aspect of the invention as do expression vector containing it. The expression vector
25 is then used in the transformation of a host cell. Culture of the transformed host cell will result in the production of the desired peptide which is then isolated.

These methods, together with the transformed
30 host cell form yet further aspects of the invention.

For instance, a peptide which comprise a recombinant hybrid protein may also be expressed with other genes, to provide a recombinant protein fused to immunogenic carrier such as the cholera toxin B subunit.
35 Conveniently, the hybrid protein will be inserted in

appropriate reading frame with the fused gene and under the regulatory control of the regulatory system of the fused gene.

The peptide of the invention obtained by recombinant technology may be glycosylated, partially glycosylated, or unglycosylated, depending on the nature of the expression host. Generally, prokaryotes such as *E. coli* will provide no glycosylation of the translated products, while yeast and mammalian cell culture will provide partial or substantial glycosylation. In regard to these different expressions possibilities, the hybrid construction may be cloned for example in pHIL D2 and pPIC 9 for its expression in the yeast, and in pCR III for its expression in mammalian cells. When employing a yeast host, codons which appear at high frequency in the structural genes encoding the yeast glycolytic enzymes may be employed, along with appropriate promoters. In some instances, it may be desirable to add or modify nucleotides in the constructions, to increase stability, enhance immunogenicity or facilitate the conjugation of the hybrid protein to a carrier. Also, the construction may include a secretory leader as processing signal sequence to effect gene product in the yeast or mammalian cells.

The peptide of the invention may be suitable for vaccine use, for which use, it will suitably be formulated as a pharmaceutical composition.

The invention further provides a pharmaceutical composition comprising a peptide as described above in combination with a pharmaceutically acceptable excipient such as carrier, liposomes or adjuvant. Suitable carriers include solid and liquid carriers such as water or saline. Alternatively the peptide may be encapsulated using conventional encapsulation methods. Other carriers and components of pharmaceuti-

cal compositions may be included as is recognized in the art.

The compositions are suitably in a form which is suitable for oral or parenteral administration.

5   The vaccine may comprise a peptide as described above itself in the form of a pharmaceutical composition.

Alternatively, a nucleic acid sequence which encodes such a peptide as described above may be incor-
10   porated into a suitable vector such as a virus vector, and this vector administered to the host in such a way that the peptide is expressed. These nucleic acid as well as vector incorporating it and pharmaceutical composition comprising said vector form a further aspect
15   of the invention.

Therefore the invention further provides a method for stimulating an immune response which method comprises administering to a patient an effective amount of a peptide as described above.

20   The method can be used for the prophylaxis or therapeutic treatment of a disease.

Furthermore the peptide of the invention will interact with antibodies specific for particular pathogenic epitopes. This has implications in the field of
25   diagnostics where the presence of an antibody specific for a particular epitope is to be determined.

The invention further provides a method for detecting the presence of an antibody to an epitope which method comprises contacting a sample suspected of
30   containing said antibody to a peptide as described above, and detecting antibody bound to said peptide.

The precise techniques used in the methods are those which are conventional in the art and include immunoassay techniques such as radioimmunoassay and
35   enzyme-linked serological assay techniques (ELISA).

For example, the peptide may be immobilized upon a support before being contacted by the sample and bound antibody detected using a labeled detection assay. Immunoassays of this type are part of the present
5   invention.

The invention further provides a diagnostic kit for use in these methods which kits comprise a peptide in accordance with the present invention.

A particular application of the invention
10 relates to the development of vaccines for *Chlamydia trachomatis*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A to 1C illustrate the hybrid construc-
15 tion coding for VDI B-T-VDIV K;

Fig. 2 illustrates the expression of the VDI B-T-VDIV K hybrid construction in pET 3b in an experiment in which *E. coli* DE3 were stimulated for expression of the recombinant protein with IPTG;

20   Fig. 3 shows the results of antibody reactivity on *E. coli* TB1 expressing the VDI B-T-VDIV K hybrid construction;

Fig. 4 shows the reactivity profile of six strains of mice immunized with the recombinant hybrid
25 protein;

Figs. 5A to 5F show the results of direct-binding radioimmunoassay measurements of specific vaginal IgA on synthetic peptides 4 VDI B and 4 VDIV K as well as on elementary bodies (EB) of serotype B and K on six
30 inbred strains of mice;

Figs. 6A to 6F show the results of direct-binding radioimmunoassay measurements of specific serum IgA on synthetic peptides 4 VDI B and 4 VDIV K as well as on elementary bodies (EB) of serotype B and K on six
35 inbred strains of mice;

Figs. 7A to 7F show the results of direct-binding enzyme-linked immunosorbent assay measurements of specific serum IgG on synthetic peptides 4 VDI B and 4 VDIV K as well as on elementary bodies (EB) of serotype
5   B and K on six inbred strains of mice.; and

Figs. 8A to 8F show the results of direct-binding enzyme-linked immunosorbent assay measurements of specific vaginal IgG on synthetic peptides 4 VDI B and 4 VDIV K as well as on elementary bodies (EB) of sero-
10   type B and K on six inbred strains of mice.

DETAILED DESCRIPTION OF THE INVENTION

Chlamydia trachomatis, an obligate intracellu-
lar bacterium, is a leading cause of sexually transmit-
15   ted diseases in industrialized countries and is also a
major cause of preventable blindness in underdeveloped
countries. It is estimated that over 300 millions of
people are infected worldwide by this microorganism.
Moreover, 55 millions of cases appear each year.
20   Based   on   pathogenicity,   antigenicity   and
nucleic acid composition, human pathogen strains of *C.
trachomatis* have been subdivided into 15 serovars. The
L1, L2, and L3 serovars are responsible for the
lymphogranuloma venereum, a relatively rare infection.
25   The A, B, Ba, and C serovars provoke the trachoma,
which   has   a   strong   incidence   in   underdeveloped
countries, while the serovars D through K are
responsible for the genital tract infections. The late
diagnostic, due to the lack of clinical symptoms, often
30   lead to severe complications such as ectopic pregnancy
and even sterility.

The first studies on human and other primates
have suggested that a vaccination using inactivated
chlamydiae frequently resulted in a more severe form of
35   trachoma following reinfection. The characterization

of the antigen implicated in the pathogenic response revealed that this antigen (the 57-kDa protein) was common to the three Chlamydia species and that it was associated with elementary bodies as well as reticulate
5 bodies. The 57-kDa protein, provokes an ocular delayed hypersensitivity reaction in animals and is implicated as mediator of the pathogenic response associated with severe chlamydial infections. The histopathology of the inflammatory response stimulated by the 57-kDa is
10 comparable to that found in severe trachoma cases and in post-salpingitis infertility.

Although the 57-kDa has been implicated in the chlamydial infections pathogenesis, its precise role in the stimulation of pathogenic responses of human dis-
15 eases has not been yet determined. However, studies have demonstrated a correlation between the capability to develop an immune response against this protein and severe infections. A correlation has been observed between the antibody response to that protein and acute
20 salpingitis and ectopic pregnancy. An association between the disease and an anti-57kDa antibody response has also been observed in other studies implicating patients suffering of tubal infertility.

These observations clearly indicate that vacci-
25 nation against chlamydiae cannot be achieved by using the whole bacteria. Novel strategies using only a part of the bacteria, such as synthetic peptides or recombi- nant proteins, have to be employed.

Surface-exposed outer membrane constituents of
30 chlamydial elementary bodies (EBs) are the likely tar- gets of the immune responses that prevent reinfection. The predominant constituents of the outer membrane are the cysteine-rich major outer membrane protein (MOMP), the cysteine-rich 60-kDa and 12-kDa proteins, and
35 lipopolysaccharides (LPS). Only the MOMP and LPS are

immunoaccessible on the EB surface, as defined by anti-body reactivity. Though surface exposed, LPS do not stimulate the production of neutralizing antibodies, whereas antibodies to the MOMP are neutralizing.

5   The MOMP, an antigenically complex protein of 40 kDa that accounts for 60%, by weight, of the chlamy-dial outer membrane proteins, exhibits both unique and common antigenic moieties, which account for the serovariation among isolates of *Chlamydia trachomatis.*

10 MOMP-encoding genes of several *Chlamydia trachomatis* serovars and *Chlamydia psittaci* strains have been cloned, sequenced and compared (Baehr, *Proc. Natl. Acad. Sci. USA* 85: 4000, 1988; Stephens, *J. Bacteriol.* 169: 3879, 1987). These studies revealed that the MOMP

15 has highly conserved portions but contains four regions of substantial sequence variability, referred to as variable sequence domains (VD) I, II, III, and IV. The nucleotide and amino acid sequences of these four variable domains are the subject of the United States

20 Patent application No. 7/324,664 filed on March 17, 1989 in the name of the U.S. Department of Health and Human Services (Washington, D.C.). These four regions contain specific sequences, referred to as neutralizable epitopes, which are the targets for

25 neutralizing antibodies. They have been characterized for several *C. trachomatis* serovars and their ability to induce the production of a neutralizing immune response has been shown (Baehr, *Proc. Natl. Acad. Sci. USA* 85: 4000, 1988; Qu, *Infect. Immun.* 61: 1365, 1993;

30 Villeneuve, *Infect. Immun.* 62: 3547, 1994; Villeneuve, *Microbiol.* 140: 2481, 1994).

     Using overlapping synthetic peptides corre-sponding to the entire MOMP sequence in T-cell prolif-eration assays and as *in vivo* priming immunogens for

35 the production of an anamnestic IgG antibody response,

Su et al. demonstrated that amino acid residues 106-130 of the MOMP possessed functional T-helper activity. This region of the MOMP is largely sequence invariant among the different *C. trachomatis* MOMP suggesting that

5   the T-helper cell epitope contained within its sequence is antigenically conserved across serovars (Su, *J. Exp. Med.* 175: 227, 1992). This conserved T-helper stimulating epitope, synthesized colinearly with B-cell neutralizing antibody stimulating epitope, has been

10   used to produce an immune response to *C. trachomatis* in a vertebrate (International Patent Application No. WO 94/06827, published on March 31, 1994 in the name of The Government of the United States of America). The peptides of WO 94/06827 were only shown to be immuno-

15   genic enough to cause the production of serum antibodies, but were not shown to elicit the production of mucosal IgA antibodies, which would be a great advantage for an effective vaccine against Chlamedia.

In addition, one region encompassing the non-

20   surface exposed VDIII has been reported to elicit T-cell help *in vivo* for the production of antibodies to protective B-cell epitopes (Allen, *J. Immunol.* 147: 674, 1991) and a T-cell epitope recognized by the majority of naive human volunteers, adjacent to VDIII

25   has also been described (Stagg, *Immunology* 79: 1, 1993).

Therefore, there is a need for a peptide or protein vaccine capable of generating a protective immune response against a variety of *C. trachomatis*

30   serovars and peptides of the invention fulfill this function.

Hence in a preferred embodiment of the peptide of the invention, the said pathogen is *C. trachomatis* or a serovar thereof.

Suitably the B-cell and T-cell epitopes which form the basis of the peptide constitute part of the major outer membrane protein of *C. trachomatis*.

Thus in a particularly preferred embodiment,
5   the present invention provides a peptide which is capable of inducing an immunological response to *C. trachomatis* comprising at least one T-helper stimulating epitope from the major outer membrane protein of *C. trachomatis* and at least two B-cell neutralizing antibody
10   body stimulating epitope from the major outer membrane protein of *C. trachomatis*.

Suitably, this peptide comprises a recombinant hybrid protein.

In a preferred embodiment of this invention,
15   the T-helper cell stimulating epitope and the B-cell neutralizing antibody stimulating epitopes are colinear. Preferably the T-helper stimulating epitopes are located within the sequence

ALNIWDRFDVFCTLGATTGYLKGNS (SEQ ID NO:1); or
20   TINKPKGYVGKEFPLDLTAGTDAAT (SEQ ID NO:2).

Preferably the B-cell neutralizing antibody stimulating epitopes are located within the sequence

LDVTTLNPTI (SEQ ID NO:3)(referred to as VDIV K); or
25   TTTGNAVAPS (SEQ ID NO:4)(referred to as VDI B).

Preferably the T-helper stimulating epitope is located in the middle of the construction, bordered to its left with the sequence VDI B and to its right with the sequence VDIV K.
30   In another preferred embodiment of this invention, the T-helper stimulating epitope is located on the protein N-terminus side of the B-cell neutralizing antibody stimulating epitopes, or on their C-terminus side, or in a combination that enhances immunogenicity.

Suitably, a linker sequence is provided so as to separate the sequences ALNIWDRFDVFCTLGATTGYLKGNS (SEQ ID NO:1) or TINKPKGYVGKEFPLDLTAGTDAAT (SEQ ID NO:2) from the sequences LDVTTLNPTI (SEQ ID NO:3) or

5   TTTGNAVAPS (SEQ ID NO:4).

It is also contemplated that the peptide additionally comprises other known or yet unknown B-cell neutralizing epitope(s) of the outer membrane protein or other known or yet unknown T-helper stimulating epi-

10   tope(s) from the major outer membrane protein of C. trachomatis or from another protein.

Further, this invention relates to a peptide comprising at least one T-helper cell stimulating epitope and at least two B-cell neutralizing antibody

15   stimulating epitopes, or isolated nucleic acid molecule coding for the said peptide, for use in preparation of a vaccine for C. trachomatis, wherein said recombinant protein of nucleic acid molecule is formulated in a pharmaceutically acceptable excipient for use as a vac-

20   cine.

This invention also relates to an immunoassay for detecting the presence of antibody to C. trachomatis in a sample, comprising a recombinant hybrid protein and means for detecting antibody bound to

25   said protein, wherein the amino acid sequence of said protein comprises at least one of the T-cell stimulating epitope and at least two of the B-cell neutralizing antibody epitopes described herein.

Peptides of the present invention may be useful

30   as substitutes for the naturally-occurring major outer membrane proteins of C. trachomatis.

The invention also relates to a peptide comprising a B-cell neutralizing antibody stimulating epitope which comprises either (a) an epitope located

35   within the sequence VDIV K (SEQ ID NO:3) or the

sequence VDI B (SEQ ID NO:4); or (b) an epitope immunologically cross-reactive with an epitope as in (a) above.

Such a peptide may be prepared and used as
5 described above in relation to the peptides comprising regions (i) and (ii).

A panel of monoclonal antibodies (mAbs) were produced, following immunizations with *Chlamydia trachomatis* whole EBs or purified MOMP. Reactivity of
10 anti-MOMP mAbs with the 15 serovars of *Chlamydia trachomatis* was further evaluated. Serovar-specific, subspecies-specific and species-specific mAbs were so purified and characterized. The *in vitro* neutralizing activity of an antibody is defined as the ability of
15 this mAb to inhibit the infection of host cells by the bacteria. The neutralizing activity of mAbs of different specificities against *Chlamydia trachomatis* serovar K, which is one of the serovar responsible of the genital tract infections, was investigated further.
20 Results indicated that five mAbs were neutralizing, one which is serovar-specific, two subspecies-specific and two species-specific (react with the 15 serovars of *Chlamydia trachomatis*).

In order to define the neutralizable epitopes
25 of the major outer membrane protein of *Chlamydia trachomatis* serovar K, fifteen peptides of ten amino acid residues, overlapping by five residues, corresponding to the four variable domains (VDI to VDIV: residues 64-85, 139-160, 224-237 and 287-319) of the
30 MOMP were synthesized. The fine specificity of seven mAbs produced (five neutralizing and two nonneutralizing mAbs) and three polyclonal antibodies, was defined by direct binding on synthetic peptides. Two neutralizable epitopes were identified, one located in
35 VDI (69SDVEGLQNSP78) and one located in VDIV

(292LDVTTLNPTI302). Neutralizable epitope located in the VDIV is a species-specific epitope of 9 uncharged residues that are conserved among all 15 serovars. This epitope has been reported by many others for its

5 ability to induce neutralizing antibodies in some *Chlamydia trachomatis* serotypes. However, such results have never been reported for serovar K.

Neutralizable epitope located in the VDI is located in the same amino acid region as a neutraliz-

10 able epitope for some C and C-related serovars such as serovars A (Baehr, *Proc. Natl. Acad. Sci. USA* 85: 4000, 1988; Su, *J. Exp. Med.* 172: 203, 1990; Su, *J. Exp. Med.* 175: 227, 1992); C, I, J, and L3 (Qu, *Infect. Immun.* 61: 1365, 1993). In order to extend these findings to

15 B-complex serovar VDI, the corresponding peptide of the VDI of serovar B, 69TTTGNAVAPS78 was synthesized. Synthetic peptides defining neutralizable epitopes (VDI-K, VDI-B and VDIV-K) were able to inhibit the binding of respective mAbs to MOMP and to whole EBs and

20 were also able to inhibit the neutralizing activity of mAbs. Moreover, mice immunized with synthetic peptides defining neutralizable epitopes, developed a humoral response against these peptides and the corresponding native EBs, and mouse sera possess neutralizing

25 activity. These data confirm the functional value of these synthetic peptides and their applicability in vaccine development.

The present invention will be more readily understood by referring to the following examples which

30 are given to illustrate the invention rather than to limit its scope.

EXAMPLE 1

Construction of the hybrid recombinant protein

Genomic DNA was extracted from purified EBs of

35 *C. trachomatis* serovars B and K using proteinase K

(100μg/ml) in 0.5% SDS, 1 hr at 37°C. Following pre-
cipitation of cell debris with CTAB/NaCl solution,
nucleic acids were phenol/chloroform/isoamyl alcohol
extracted, and isopropanol precipitated. The DNA was
5   rinsed in 70% ethanol, dried, and then suspended in
tris-EDTA (TE) buffer.

The MOMP genes fragments were obtained by
polymerase reaction amplification from genomic DNA. As
is illustrated in Fig. 1A, sense primer used for the
10   MOMP B fragment amplification was 5' G-AAA-GAA-TTC-AAA-
ATG-GGT-G 3' (SEQ ID NO:5), comprising an Eco RI diges-
tion site, and antisense primer was 3' CCT-TTG-AGA-CGA-
AGA-AAG-TTA-GAC-GTC-ATC 5' (SEQ ID NO:6), comprising a
Pst I digestion site, in order to amplify the region
15   coding for the B-cell neutralizing antibody stimulating
epitope (TTTGNAVAPS)(SEQ ID NO:4) and the T-helper
stimulating epitope (ALNIWDRFDVFCTLGATTGYLKGNS)(SEQ ID
NO:1). As is illustrated in Fig. 1B, sense primer used
for the MOMP K fragment amplification was 5' G-ATC-CGT-
20   ATC-CTG-CAG-CCT-AAA-TTG 3' (SEQ ID NO:7), comprising a
Pst I digestion site, and antisense primer was 3' CCT-
GAC-CGA-CTA-TGT-TAC-ATT-CTT-AAG-AGG 5' (SEQ ID NO:8),
comprising an Eco RI digestion site, in order to am-
plify the region coding for the B-cell neutralizing
25   antibody stimulating epitope (LDVTTLNPTI)(SEQ ID NO:3).
The amplification was carried out in a 100 μl volume
containing 100 ng of genomic DNA. A DNA Thermal Cycler
™ (Perkin-Elmer Model 9600) was used with 30 sec of
melting at 94°C, 30 sec of annealing at 50°C, and 30
30   sec of polymerization at 72°C for 35 cycles. Amplifi-
cation products of the correct size were isolated from
a 1.2% agarose gel.

The amplified ompl DNA fragments were phos-
phorylated and blunted. The plasmid vector pBluescript
35   II KS- (BS) was digested with Eco RV and Sma I, in

order to remove the Eco RI and Pst I digestion sites of
the polylinker and further alkaline phosphatase
treated. The VDI B-T fragment and the VDIV K fragment
were excised from a 1.2% agarose gel. Each one of the

5   fragments were ligated in BS, and transformed in *E.
coli* DH5α, leading to the formation of clones desig-
nated BS-VDI B-T and BS-VDIV K. Clones of both type
were further digested with Pst I and Xba I, in order to
1) release the VDIV K fragment (see Fig. 1A) and 2)

10   open the BS-VDI B-T vector (see Fig. 1B). Isolated
VDIV K fragment from Fig. 1B was then ligated in BS-VDI
B-T from Fig. 1A, resulting in BS-VDI B-T-VDIV K of
Fig. 1C, with both gene fragments in the appropriate
reading frame as shown by DNA sequencing. The hybrid

15   construction, flanked either end by an Eco RI digestion
site (Fig. 1C), was released after an Eco RI digestion,
isolated from the gel, blunted and further ligated in
blunted-pET 3b for transformation in *E. coli* BL21
(DE3).

20   **EXAMPLE 2**
                Recombinant hybrid protein expression
         The expression of the recombinant hybrid pro-
tein by DE3/pET 3b-VDI B-T-VDIV K cells was evaluated
by SDS-PAGE and Western blot analysis. Culture of

25   DE3/pET 3b-VDI B-T-VDIV K was grown overnight in LB
broth plus 100 μg of ampicillin per ml. Overnight cul-
tures were diluted 1/100 in fresh LB and grown to mid-
log phase (O.D.$_{600}\approx0.5$), and recombinant hybrid protein
expression was induced by the addition of 0.3 mM IPTG

30   (isopropyl-β-D-thiogalactopyranoside). Culture super-
natant, whole cells, cell membrane and cytoplasm were
isolated and the activity of characterized monoclonal
and polyclonal antibodies was then assessed on these
fractions by direct-binding ELISA and by immunoblot

35   analysis.

SDS-PAGE analysis shows the apparition of a protein of an expected molecular weight of approximately 16 kDa in cytoplasm (Fig. 2). Monoclonal antibody directed against the described neutralizable epi-
5   tope of the VDIV K, along with polyclonal antibody specific of the neutralizable epitope of the VDI B, were used for the characterization of the recombinant hybrid protein expressed by *E. coli*. Recognition of the expressed epitopes by their respective antibody was
10 shown by direct-binding ELISA (Fig. 3), as well as by immunoblot analysis, indicating that neutralizable epitopes were expressed in an appropriate conformation. Furthermore, knowing that antibodies used here reacted with chlamydial EBs in their native form (Villeneuve,
15 *Infect. Immun.* **62**: 3547, 1994; Villeneuve, *Microbiol.* **140**: 2481, 1994), the recognition of the recombinant hybrid protein by these antibodies confirms the relevancy of its use as a vaccine.

The recombinant hydrid proteins, as described
20 herein or in accordance to the possible modifications described previously, may also be expressed with other genes, to provide a recombinant protein fused to immunogenic carrier such as the cholera toxin B subunit. Conveniently, hydrid protein will be inserted in appro-
25 priate reading frame with the fused gene and under the regulatory control of the regulatory system of the fused gene.

The recombinant hydrid proteins may be glycosylated, partially glycosylated or unglycosylated,
30 depending on the nature of the expression host. Generally, prokaryotes such as *E. coli* will provide no glycosylation of the translated products, while yeast and mammalian cell culture will provide partial or substantial glycosylation. In regard to these different
35 expression possibilities, we have cloned the hybrid

construction in pHIL D2 and pPIC 9 for its expression in the yeast, and in pCR III for its expression in mammalian cells. When employing a yeast host, codons which appear at high frequency in the structural genes

5 encoding the yeast glycolytic enzymes may be employed, along with appropriate promoters. In some instances, it may be desirable to add or modify nucleotides in the constructions, to increase stability, enhance immunogenicity or facilitate the conjugation of the hybrid

10 protein to a carrier. Also, the constructions may include a secretory leader and processing signal sequence to effect gene product in the yeast or mammalian cells.

15   **EXAMPLE 3**

Immunogenicity of the recombinant hybrid protein
BALB/cJ (H-2d), A/J (H-2a), C57BL/10SnJ (H-2b), CBA/J (H-2k), DBA/1J (H-2q) and SJL/J (H-2s) mice were purchased from Jackson Laboratory (Bar Harbor, ME).

20 Females at 8-12 weeks of age were used for experimentations. Groups of five mice were immunized by intraperitoneal injection of 50 μg of VDI B-T-VDIV K emulsified in Complete Freund's Adjuvant (CFA) on day 0, and boosted once three weeks later with the same dose of

25 recombinant protein in Incomplete Freund's Adjuvant. Mice were bled three weeks after the secondary immunization.
A (G-17), B (TW-S), Ba (AP-2), C (TW-3), D (UW-3), E (UW-5), F (UW-55), G (UW-57), H (UW-4), I (UW-

30 12), J (UW-36), K (UW-31), L1 (440), L2 (434), and L3 (404) elementary bodies (EBs) were purified from infected cells by centrifugation on a Renografin density gradient. The presence of *C. trachomatis* specific IgG was evaluated by enzyme linked immunoabsorbent

35 assay (ELISA). This experiment was done in order to assess the functionality of the T-cell stimulating

epitope. In fact, the presence of IgG indicates that memory T-helper cells have been primed and recalled. The IgG reactivity on the 15 *C. trachomatis* serovars was therefore tested. Briefly, microtiter plates were
5   coated overnight at 4°C with 100 µl of formalin-killed EBs (5µg/ml) in phosphate-buffered saline (PBS, pH 7.4). After saturation with bovine serum albumine (3%) in PBS, sera were added at a dilution of 1/2000 for 1h30 at 37°C. After appropriate washes, mouse
10 immunoglobulin G was detected by using a biotin-labeled anti-mouse IgG (Jackson Immunoresearch Laboratories), 1h30 at 37°C. The reaction was developed in a peroxidase substrate solution [2,2'-azino-di (3-ethylbenzthiazoline sulfonate) and H_2O_2] following the
15 addition of a streptadivine-peroxidase conjugate. The A_{405} was then measured. Pooled pre-immune sera were used as the negative controls.

As shown in Fig. 4, sera from the six strains of mice that were immunized with the recombinant hybrid
20 protein reacted with several serotypes. Although the A/J response was generally weaker, results of this study are consistent with the hypothesis that the T helper cell epitope contained in the SEQ ID NO:1 is recognized by multiple MHC class II haplotypes and that
25 these T-cell determinants provide cognate help for antibody production. Also the serotypes corresponding to the sequences enclosed in the hybrid protein, or serotypes closely related (B, Ba and K, L3) were better recognized. Therefore, it is contemplated that the
30 preferred recombinant hybrid protein vaccine of this invention can additionally incorporate B-cell neutral-izing epitope in order to evoke a broadly cross reac-tive antibody response.

EXAMPLE 4
In vitro neutralization of chlamydial infectivity

Neutralization assays were done without centrifugation. Briefly, purified EBs were diluted in
5 0.25M sucrose - 10 mM sodium phosphate - 5mM L-gluamic acid (SPG) (pH 7.2) for a final concentration of 4 X 10^5 inclusion forming units (IFUs) per ml. Two fold dilutions of pooled mouse sera (diluted in SPG-0.5% decomplemented fetal calf serum (FCS)) were mixed with
10 an equal volume of chlamydiae. The mixture was incubated at 37°C for 60 minutes, and 400 µl of the appropriate *C. trachomatis*-serum mixture dilutions were inoculated in triplicate onto confluent HaK cell monolayers.

15 After 2h of incubation, the inocula were removed and the monolayers were washed and fed with 500 µl of Eagle's minimal essential medium supplemented with 10% FCS containing 0.5µg of cycloheximide per ml and incubated at 37°C for 72h. The monolayers were
20 fixed with methanol, and chlamydial inclusions were identified by fluorescent-antibody staining with a genus specific mAb. A 50% or greater reduction from the number of control inclusion-forming units in infectivity was considered positive for neutralization.

25 Immune sera from mice immunized with the recombinant hybrid protein were assayed for their *in vitro* neutralizing activity as described above. As shown in Table I, serum from mouse strains tested displayed a significant neutralizing activity against serovar K.
30 Although activity against serovar B and others remains to be tested, these results indicate that antibodies generated in animals, in response to the recombinant hybrid protein, were functional neutralizing antibodies and that this protein constitutes a very attractive
35 vaccine structure.

Table 1
In vitro neutralization of *Chlamydia trachomatis* serovar K by immune sera from mice immunized with the recombinant hydrid protein

MICE IMMUNIZED WITH VDI B-T-VDIV K	NEUTRALIZING TITER*	
	Day 0	Day 42
C57BL/10SnJ	0	200
CBA/J	0	800

* Neutralizing titers are expressed as the reciprocal of pooled serum dilutions that neutralized 50% of chlamydial infectivity. Preimmune or normal sera had neutralizing titers of less than 20.

EXAMPLE 5
Induction of a Chlamydia-specific mucosal response

The local response has a critical role to play to prevent invasion of the host by organisms, such as *Chlamydia trachomatis*, penetrating through the mucosa. The importance of secretory IgA antibodies has been clearly shown but cellular immunity is probably also involved particularly in the view that *Chlamydia trachomatis* is an intracellular organism. In fact, the production of IgA reflect activation of both B and T cells. The development of such a protective immune response in the mucosa is the target of the Chlamydia vaccine program. However, this objective should be achieved with the concern that the vaccine has to be designed for human use. Therefore, adjuvants, carriers or vectors acceptable by the human regulatory agencies were used in the development of this vaccine.

The objectives of this experiment were to induce, following intra-nasal immunization with repetitive synthetic peptides or the recombinant hybrid protein, alone or in combination with the cholera toxin B subunit (CTB) as "adjuvant", a specific mucosal IgA

antibody response. Also, to analyze the MHC restric-
tion of the induced immune response by using strains of
mice differing at the H-2 locus. Finally, to assess
the functionality of this antibody response and its
5 ability to neutralize infectivity in vitro, which is an
essential criteria for the development of an effective
vaccine.

Briefly, BALB/cJ (H-2d), A/J (H-2a), C57BL/10
SnJ (H-2b), CBA/J (H-2k), DBA/1J (H-2q) and SJL/J (H-
10 2s) mice were purchased from Jackson Laboratory (Bar
Harbor, ME). Females at 8-12 weeks of age were used
for experimentations and did not receive any hormonal
treatment. Groups of 8 mice were immunized intra-
nasally with 20 µg of cholera toxin B subunit (CTB), 25
15 µg 4 VDI B + 25 µg 4 VDIV K with or without 20 µg of
CTB, or 50 µg of rVDI B-T1-VDIV K with or without 20 µg
of CTB. Antigens were delivered to mice by introducing
10 µl of PBS in each nostril using a micropipette.
Intra-nasal immunizations were carried out on days 0,
20 14, 28 and 62. Bleeding and vaginal washes (2 X 30µl)
were performed on days 1, 13, 27, 41, 55, 69 and 75.
Sera were decomplemented by heating 30 minutes at 56°C.
Vaginal washes were centrifuged and supernatants, as
well as sera, were stored at -70°C until tested.
25 Specific serum and vaginal IgA were monitored
by solid-phase radioimmunoassay (RIA). For specific
IgA, microtiter plates were coated overnight at 4°C
with 100µl of EBs (5µg/ml) of serotype B or K in PBS
(pH 7.4), or with synthetic peptides (50µg/ml) 4 VDI B,
30 4 VDIVK (corresponding to four-times repeats of target
epitopes) or irrelevant control peptide (from the
sequence of the allergen Lolium perenne) in 50 mM car-
bonate buffer (pH 9.6). After saturation with BSA (3%)
in PBS, appropriate dilutions of pooled sera or vaginal
35 washes were added for 1h30 at 37°C. After washes,

mouse immunoglobulin A were detected by using a ^{125}I-anti-mouse IgA (Pierce Biochemical Co., USA), 1h at room temperature. Radioactivity was measured on a 1261 Multigamma™ counter (LKB Wallace). Non specific bind-

5   ing of vaginal washes and sera on an irrelevant peptide was less than 1 and 10% respectively.

Total vaginal IgA was also monitored by solid-phase RIA. Briefly, microtiter plates were coated overnight at 4°C with 100 µl of a sheep anti-mouse IgA

10   (The Binding Site, Birmingham, England) (10µg/ml) in 50 mM carbonate buffer (pH 9.6). After saturation with BSA (3%) in PBS, appropriate dilutions of pooled sera or vaginal washes were added for 1h30 at 37°C. Puri-fied mouse IgA was used for the standard curve and was

15   added to the anti-IgA antibody in the same conditions. After washes, mouse immunoglobulin A were detected as described above.

Results were reported in nanograms of Chlamy-dia-specific IgA per total IgA (micrograms per millili-

20   ter) to minimize differences between groups due to variability in total immunoglobulin concentrations resulting from the vaginal washes.

Specific serum and vaginal IgG responses were further monitored by enzyme-linked immunosorbent assay.

25   Briefly, microtiter plates were coated overnight at 4°C with 100 µl of EBs (5µg/ml) of serotype B or K in PBS (pH 7.4), or with synthetic peptides (50µg/ml) 4 VDI B, 4 VDIV K or irrelevant control peptide in 50 mM carbon-ate buffer (pH 9.6). After saturation with BSA (3%) in

30   PBS, appropriate dilutions of pooled sera or vaginal washes were added for 1h30 at 37°C. After appropriate washes, mouse immunoglobulin G were detected by using a peroxidase-conjugated goat anti-mouse IgG. The reac-tion was developed in a peroxidase substrate solution

35   [2,2'-azino-di(3-ethylbenzthiazoline sulfonate) and

H_2O_2]. The A_{405} was then measured. Non specific binding of vaginal washes and sera on an irrelevant peptide gave O.D. of less than 0.15 and 0.5 respectively.

Complement-independent neutralization assays were carried out without centrifugation. Briefly, purified EBs were diluted in 25 µl of 0.25 M sucrose/10 mM sodium phosphate/5mM L-glutamic acid (SPG, pH 7.2) to give a final concentration of 6 X 10^4 inclusion-forming-units (IFUs) per ml. The bacteria were then mixed with an equal volume of serially diluted pooled vaginal washes (diluted in SPG plus 0.5% fetal calf serum). The mixture was incubated at 37°C for 60 min an further diluted to give a final volume of 400 µl, before to be inoculated onto confluent HaK cell monolayers grown in 24-well plates (Costar). After 2h incubation, the inocula were removed and the monolayers washed with Hank's balanced salts solution. Monolayers were fed with 500 µl of Eagle's minimal essential medium supplemented with 10% fetal calf serum containing 0.5 µg cycloheximide/ml and incubated at 37°C, 5% CO_2 for 72h. The monolayers were fixed with methanol, and chlamydial inclusions were identified by indirect fluorescent antibody staining using a genus-specific mAb to chlamydial LPS. The total number of inclusions per well were counted. Samples were tested in duplicate.

Groups immunized with the CTB alone or with the repetitive synthetic peptides, alone or in combination with the CTB, did not produce significant amounts of specific vaginal or serum IgA. However, as shown in Fig. 5, BALB/c mice immunized with the recombinant hybrid proteins developed significant amounts of specific vaginal IgA antibodies. These antibodies efficiently recognized the VDIV K epitope in its synthetic form, and to a lesser extent the native epitope on the

EB of *Chlamydia trachomatis* serotype K.   The IgA also
weakly reacted with the synthetic peptide 4 VDI B,
although the latter response was less significant.   The
response occurred following the fourth immunization on
5   day 62.   Surprisingly, mice which received the recombi-
nant hybrid protein along with the CTB, did not develop
Chlamydia-specific IgAs.   To date however, the efficacy
of CTB as a mucosal adjuvant remains equivocal.
Indeed, the CTB has been reported to enhance the immune
10   response to some mucosally administered antigens, while
failing to have such an effect to other antigens unless
holotoxin was added.   The immunogenicity of antigens
was reported to be better when they were conjugated to
CTB instead of simply mixed together with it.

15   The Chlamydia-specific vaginal IgA response was
also studied in other strains of mice, varying at the
H-2 haplotype of MHC class II.   Although slight vari-
ations can be observed among the different strains of
mice, a significant specific IgA antibody response was
20   seen at mucosal site in all strains.   Results showed
that some strains of mice developed a better response
to the VDIV epitope (BALB/c and C57BL/10 SnJ), while
for other strains, no significant differences can be
seen between the reactivity for the VDI B and the VDIV
25   K epitopes (A/J, DBA/1J, and SJL/J).   It is important
to note that levels of mucosal IgA antibodies against
the native form of the epitopes (elementary bodies), as
well as their synthetic forms, were comparable except
for BALB/c where higher anti-peptide responses were
30   observed and CBA/J where a strong anti-VDI B response
can be seen.

          In some strains of mice (BALB/c, CBA/J and
DBA/J), an increase in the IgA antibody production fol-
lowing the last immunization on day 62 was observed.

On another hand, the specific serum IgA response (Fig. 6), although detectable, was around ten fold weaker than the vaginal response, suggesting that IgA in the vaginal fluids does not originate from serum
5 transudation through the vaginal epithelium.

As mentioned for the BALB/c, the IgA antibody response observed for mice immunized with the recombinant protein along with the CTB was lower or comparable to the one observed for mice immunized with rVDI B-T1-
10 VDIV K alone. Higher levels of mucosal IgA were not seen in any of the mouse strains analyzed.

As shown on Fig. 7, mice immunized intranasally with rVDI B-T1-VDIV K also generated a significant serum IgG response. The levels of specific IgG
15 increase over time and seem to remain stable, in contrast to the vaginal IgA. This indicates that in addition to the induction of a mucosal IgA response, immunization with the recombinant hybrid protein led to the production of specific serum IgG. It is interesting to
20 note that the peak in IgG and IgA response often coincided further confirming the memory type of these responses. This is well illustrated in the case of BALB/c, where a strong IgA and IgG anti-4 VDIV K response developed after the last immunization on day
25 62.

Groups immunized with the CTB alone or with the repetitive synthetic peptides, alone or in combination with the CTB, did not develop a significant specific serum IgG response. However, mice immunized with the
30 CTB in addition to rVDI B-T1-VDIV K developed IgG antibodies. Finally, no specific IgG were detectable in the vaginal washes (Fig. 8).

Preliminary results suggest that the specific IgA antibodies found in vaginal washes were functional.
35 Indeed, C57BL/10 SnJ mice immunized with the rVDI B-T1-

VDIV K protein developed an *in vitro* neutralizing activity against *C. trachomatis* serovar K. A 39% reduction of chlamydial infectivity was observed when using vaginal washes harvested at day 55 following immunization. Such an inhibition was not observed with vaginal washes obtained from non immunized mice. These data are promising taking into account the dilution factor in the vaginal washes. Indeed, the Inventor reported that 3 µg/ml of anti-*C. trachomatis* mAb (DP10) were required to obtain a 50% reduction of chlamydial infectivity; these concentrations are higher than those measured in the vaginal washes.

These results suggest that a Chlamydia-specific vaginal IgA response was achieved upon nasal immunization with the recombinant hybrid protein and without the use of any adjuvant. Moreover, the induced vaginal IgA response was functional since it significantly reduced the *in vitro* chlamydial infectivity. Finally, results showed that the helper T-cell epitope of the recombinant hybrid protein was recognized in the context of multiple MHC haplotypes, since the responses were observed in all strains of mice. A Chlamydia-specific serum IgG response was also seen.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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    (ii) TITLE OF INVENTION: NOVEL PEPTIDE AND ITS USE AS VACCINE

    (iii) NUMBER OF SEQUENCES: 8

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: GB 951623.9
        (B) FILING DATE: 09-AUG-1995

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Ala Leu Asn Ile Trp Asp Arg Phe Asp Val Phe Cys Thr Leu Gly Ala
1               5                   10                  15

Thr Thr Gly Tyr Leu Lys Gly Asn Ser
        20                  25
```

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Thr Ile Asn Lys Pro Lys Gly Tyr Val Gly Lys Glu Phe Pro Leu Asp
1   5   10   15

Leu Thr Ala Gly Thr Asp Ala Ala Thr
            20   25

(2) INFORMATION FOR SEQ ID NO: 3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 10 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

       (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Leu Asp Val Thr Thr Leu Asn Pro Thr Ile
1   5   10

(2) INFORMATION FOR SEQ ID NO: 4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 10 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

       (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Thr Thr Thr Gly Asn Ala Val Ala Pro Ser
1   5   10

(2) INFORMATION FOR SEQ ID NO: 5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 20 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "Sense DNA Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GAAAGAATTC AAAATGGGTG

                                        20

(2) INFORMATION FOR SEQ ID NO: 6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "Anti-Sense DNA Primer"

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTACTGCAGA TTGAAAGAAG CAGAGTTTCC   30

(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "Sense DNA Primer"

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GATCCGTATC CTGCAGCCTA AATTG   25

(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "Anti-Sense DNA Primer"

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGAGAATTCT TACATTGTAT CAGCCAGTCC   30

I CLAIM:

1.   A recombinant hybrid protein comprising one or several T-helper cell stimulating epitope from the major outer membrane protein of *C. trachomatis* and two or more B-cell neutralizing antibody stimulating epitopes from the major outer membrane protein of *C. trachomatis*.
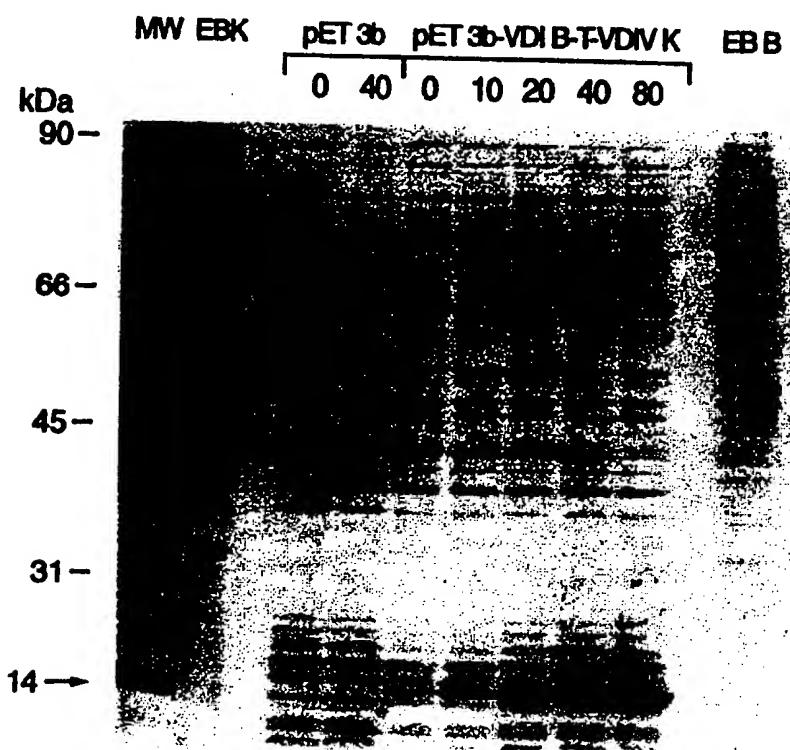
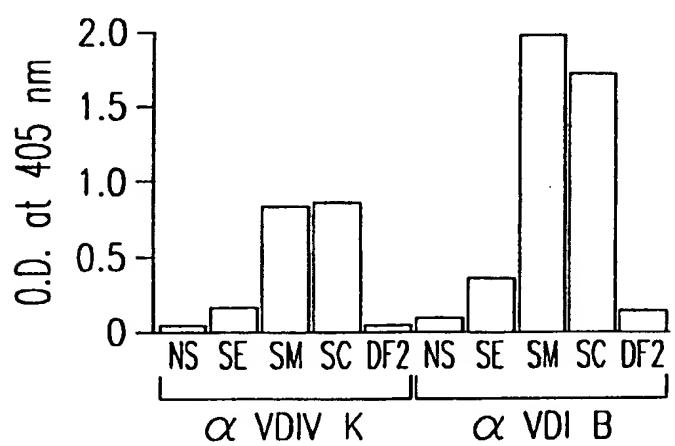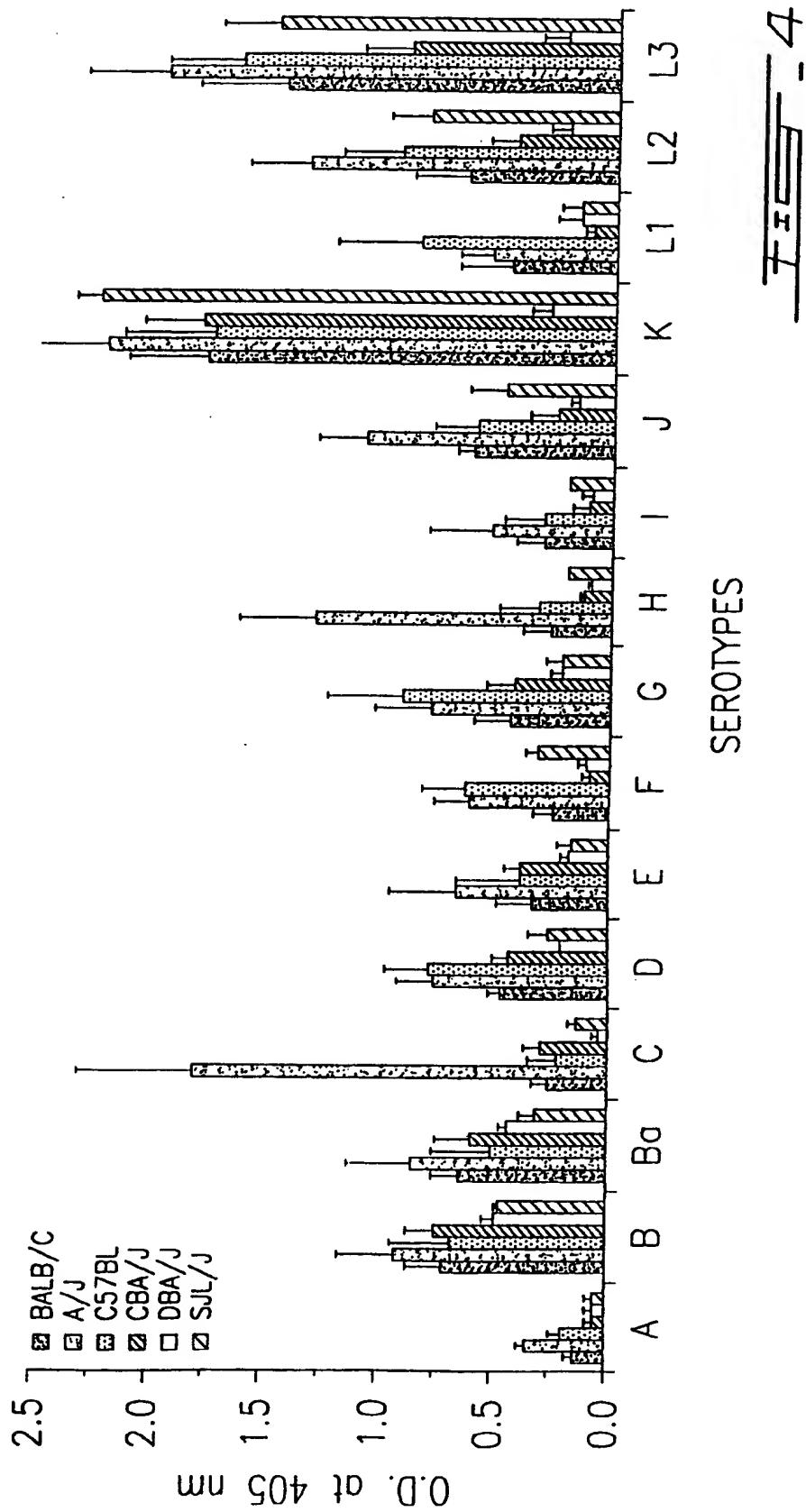2.   The recombinant hybrid protein of claim 1, wherein said T-helper cell stimulating epitope comprises the amino acid sequence SEQ ID NO:1 or SEQ ID NO:2.

3.   The recombinant hybrid protein of claim 1, wherein said B-cell neutralizing antibody stimulating epitope comprises the amino acid sequence SEQ ID NO:3 or SEQ ID NO:4.

4.   The recombinant hybrid protein of claim 2 or 3, wherein said T-helper stimulating epitope is either flanked by said B-cell neutralizing antibody stimulating epitopes, or on their N-terminus side, or on their C-terminus side, or in a combination that enhances immunogenicity.

5.   The recombinant hybrid protein of claim 4, further comprising a linker sequence inserted between the sequences SEQ ID NO:1 or SEQ ID NO:2 from the sequences SEQ ID NO:3 or SEQ ID NO:4.

6.   The recombinant hybrid protein of Claim 4, wherein said protein further comprises at least one other T-helper stimulating epitope from a protein other than the outer membrane protein of *C. trachomatis*.

7.   A nucleic acid molecule coding for the expression of the recombinant hybrid protein of claim 1.

8.   Use of a recombinant hybrid protein according to claim 1 for the preparation of a vaccine for C. trachomatis.

9.   Use of a nucleic acid molecule according to claim 7 for the preparation of a vaccine for C. trachomatis.

10.   Use of the recombinant hybrid protein according to claim 1 in an immunoassay for detecting the presence of antibodies to C. trachomatis in a sample.

11.   Composition comprising a recombinant hybrid protein according to claim 1, 2, 3, 4, 5 or 6 in association with a pharmaceutically acceptable excipient.

12.   Use of a composition according to claim 11 as a vaccine for C. trachomatis.

13.   A method for preparing a recombinant hybrid protein according to claim 1, 2, 3, 4, 5 or 6, which comprises the step of synthesizing an amino acid sequence, wherein said amino acid comprises an antigenic determinant or target epitope in a repetitive form.

14.   Method for stimulating an immune response, said method comprising the step of administering to a patient an effective amount of a recombinant hybrid protein according to claim 1, 2, 3, 4, 5 or 6.

15.   The method of claim 14, for the prophylaxis or treatment of a disease.

16.   Method for detecting the presence of an antibody to an epitope, said method comprising the steps of i) contacting a sample suspected of containing said antibody to a recombinant hybrid protein according to claim 1, 2, 3, 4, 5 or 6, and ii) detecting said antibody bound to said peptide.

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17.   A diagnostic kit for use in a method according to claim 16, said kit comprising a recombinant hybrid protein comprising one or several T-helper cell stimulating epitope from the major outer membrane protein of *C. trachomatis* and two or more B-cell neutralizing antibody stimulating epitope from the major outer membrane protein of *C. trachomatis*.

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Serotype B *omp1*

```
         EcoR I      VDI B-T1
       GAAAGAATTCAAAATGGGTG                            GGAAACTCTGCTTCTTTCAATTTAGTGGGG
       TAAAGAATTCCAAATGGGTG.........                   CCTTTGAGACGAAGAAAGTTAAATCACCCC
       ATTTCTTAAGGTTTACCCAC.........                   CCTTTGAGACGAAGAAAGTTAGACGTCATC
                                                                             Pst I
```

Xba I

pBluescript II KS (-)-
VDI B-T1

Lac I

Amp

Fig. 1a

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Serotype K *omp1*

```
            Pst I        VDIV K
GATCCGTATCCTGCAGTCCTAAATTG.
CTAGGCATAGGACGTCGGATTTAAC.......
GATCCGTATCGCTCAGCCTAAATTG.........GAACTGGCTGATACAATGCAAATCGTTTCC
CTAGGCATAGGACGTCGGATTTAAC.........CTTGACCGACTATGTTACGTTTAGCAAAGG
                                  CCTGACCGACTATGTTACATTCTTAAGAGG
                                                          EcoR I
```

pBluescript II KS (-)-
VDIV K

Lac I

Xba I

Amp.

FIG. 1B

FIG. 1c

FIG. 2

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FIG. 3

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FIG. 4

SEROTYPES

O.D. at 405 nm

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FIG_5A

FIG_5B

FIG_5C

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FIG. 5D

FIG. 5E

FIG. 5F

FIG. 6A

FIG. 6B

FIG. 6C

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FIG 6D

FIG 6E

FIG 6F

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Figure 7A (BALB/c)

Figure 7B (A/J)

Figure 7C (C57BL/10 SnJ)

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FIG._7D

FIG._7E

FIG._7F

FIG. 8A

FIG. 8B

FIG. 8C

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/62   C07K19/00   C07K14/295   A61K39/118   C12P21/02
G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,94 06827 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 31 March 1994 cited in the application SEQ ID Nos. 1-3; pages 5-7, page 14, second paragraph ---	1-4,6-17
Y	VACCINE, vol. 11, no. 11, August 1993, page 1159-1166 XP000608497 SU, H. AND CALDWELL, H.D.: "Immunogenicity of a synthetic oligopeptide corresponding to antigenically common T-helper and B-cell neutralizing epitopes of the major outer membrane protein of Chlamydia trachomatis" page 1160, right-hand column, fourth paragraph; page 1165, left-hand column second paragraph ---	1-4,6-17

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| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
11 November 1996	29. 11. 96

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Inter.   nal Application No

PCT/CA 96/00538

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 175, - January 1992 pages 227-235, XP000607584 SU, H. AND CALDWELL, H.D.: "Immunogenicity of a chimeric peptide corresponding ro T helper and B cell epitopes of the Chlamydia trachomatis major outer membrane protein" cited in the application page 228, "Synthetic Peptides"; Tables 1 and 2 ---	1-4,6-17
Y	VACCINE, vol. 12, no. 6, May 1994, pages 557-564, XP000608457 QU, Z. ET AL.: "Analysis of the humoral response elicited in mice by a chimeric peptide representing variable segments I and IV of the major outer mebrane protein of Chlamydia trachomatis" page 561, right-hand column, second paragraph; page 563, left-hand column ---	1-4,6-17
Y	INFECTION AND IMMUNITY, vol. 62, no. 8, August 1994, pages 3547-3549, XP000608433 VILLENEUVE, A. ET AL.: "Characterization of the humoral response induced by a synthetic peptide of the major outer membrane protein of Chlamydia trachomatis serovar B" cited in the application see page 3547, left-hand column, second paragraph and right-hand column, lines 14-15 ---	1,3,4, 6-17 ·
Y	MICROBIOLOGY, vol. 140, 1994, pages 2481-2487, XP000607586 VILLENEUVE, A. ET AL.: "Determination of neutralizing epitopes in variable domains I and IV of the major outer-membrane protein from Clamydia trachomatis serovar K" cited in the application see page 2483, right-hand column, lines 7 and 25; page 2485, second paragraph ---	1,3,4, 6-17

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 172, July 1990, pages 203-212, XP000607583 SU, H. ET AL.: "Identification and characterization of T helper cell epitopes of the major outer membrane protein of Chlamydia trachomatis" Figure 2, "A8" and "A15"; page 208, right-hand column, lines 4-11; page 210, left-hand column	1,2,4, 6-17
Y	IMMUNOLOGY, vol. 79, 1 May 1993, pages 1-9, XP000607575 STAGG, A.J. ET AL.: "Primary human T-cell responses to the major outer membrane protein of Chlamydia trachomatis" cited in the application Figure 6, "P11"; Table 2, "P11"; page 7, lines 9-13	1,2,4, 6-17
A	EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 22, no. 10, October 1992, pages 2675-2680, XP000607498 PARTIDOS, C. ET AL.: "The influence of orientation and number of copies of T and B cell epitopes on the specificity and affinity of antibodies induced by chimeric peptides" the whole document	1
A	INFECTION AND IMMUNITY, vol. 57, no. 2, February 1989, pages 487-494, XP000608912 CARLSON, E. J. ET AL.: "Coning and characterization of a Chlamydia trachomatis L3 DNA fragment that codes for an antigenic region of the major outer membrane protein and specifically hybridizes to the C-and C-related complex serovars" see the whole document	1
A	WO,A,94 26900 (CONNAUGHT LABORATORIES LIMITED) 24 November 1994 the whole document, especially page 3, lines 14-18	1

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page 3 of 3

Inte: .nal Application No

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9406827	31-03-94	AU-B-	669000	23-05-96
		AU-A-	4925093	12-04-94
		CA-A-	2144882	31-03-94
		EP-A-	0662083	12-07-95
		JP-T-	8504188	07-05-96
WO-A-9426900	24-11-94	AU-A-	6718394	12-12-94
		CA-A-	2162664	24-11-94
		EP-A-	0698100	28-02-96